

Effect of different irrigation solutions and calcium hydroxide on bacterial LPS

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Abstract

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Aim To evaluate the effect of biomechanical preparation with different irrigating solutions and calcium hydroxide dressing in dog root canals containing bacterial endotoxin (lipopolysaccharides; LPS).

Methodology One hundred and forty premolar roots from seven dogs were filled with *Escherichia coli* LPS for 10 days (three roots were lost during histological processing). The following irrigating solutions were used for biomechanical preparation: 1% (group I, $n = 20$), 2.5% (group II, $n = 19$) and 5% sodium hypochlorite (group III, $n = 19$), 2% chlorhexidine digluconate (group IV, $n = 20$) and physiological saline solution (group V, $n = 19$). In group VI ($n = 20$), the LPS solution was maintained in the root canal during the entire experiment and in group VII ($n = 20$), after biomechanical preparation with saline solution, the root canals were filled with

a calcium hydroxide dressing (Calen; control). After 60 days, the animals were sacrificed and the following parameters of periapical disease were evaluated: (a) inflammatory infiltrate, (b) periodontal ligament thickness, (c) cementum resorption and (d) bone resorption. Scores were given and data were analysed statistically with the Kruskal–Wallis and Dunn tests ($P < 0.05$).

Results Histopathological evaluation showed that groups I–VI had more inflammatory infiltrate, greater periodontal ligament thickening and greater cementum and bone resorption ($P < 0.05$) compared to group VII, which received the calcium hydroxide intracanal dressing.

Conclusions Biomechanical preparation with the irrigating solutions did not inactivate the effects of the endotoxin but the calcium hydroxide intracanal dressing did appear to inactivate the effects induced by the endotoxin *in vivo*.

Keywords: calcium hydroxide, chlorhexidine, endodontics, endotoxin, sodium hypochlorite.

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Introduction

Currently, microbial factors are considered the main cause of pulp and periapical disease (Takehashi *et al.* 1965). In 1970s, endodontic microbiological studies reported that non-vital teeth with radiographically visible chronic periapical lesions showed a predominance of anaerobic Gram-negative microorganisms (Sundqvist 1976, Assed *et al.* 1996, Abou-Rass & Bogen 1998). Gram-negative microorganisms produce products and

subproducts that are toxic to periapical tissues and have lipopolysaccharides (LPS) within their cell wall that are released during duplication or bacterial death and adhere irreversibly to mineralized tissue (McGee *et al.* 1992, Barthel *et al.* 1997).

Lipopolysaccharides stimulate macrophages to release cytokines such as tumour necrosis factor (McGee *et al.* 1992, Barthel *et al.* 1997) and interleukins 1, 6 and 8 (Matsushita *et al.* 1999) that are important for the initial process and maintenance of the inflammatory response and periapical bone resorption (Dwyer & Torabinejad 1981, Pitts *et al.* 1982, Rietschel & Brade 1992).

Therefore, the aim of root-canal treatment of teeth with pulp necrosis and chronic periapical lesions is based not only on eliminating microorganisms and

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substrate but also on inactivating toxic effects of LPS, which is important for the healing of periapical tissues (Silva *et al.* 2002). However, biomechanical preparation of the root canal only partially reduces the endodontic microbiota, but does not eliminate bacteria from the entire system (Beruti *et al.* 1997), and does not act on LPS (Buttler & Crawford 1982, Buck *et al.* 2001). Thus, the use of an intracanal dressing is considered as necessary by many (Leonardo *et al.* 1995, Assed *et al.* 1996, Tanomaru Filho *et al.* 2002).

Inactivation of LPS by calcium hydroxide was shown in *in vitro* studies (Safavi & Nichols 1993, 1994, Barthel *et al.* 1997, Olsen *et al.* 1999). Silva *et al.* (2002) showed that LPS did not induce chronic periapical lesions in dog's teeth when combined in root canals with calcium hydroxide.

The aim of this study was to evaluate the effect of different irrigating solutions used during biomechanical preparation and calcium hydroxide used as an intracanal dressing on LPS-induced periapical inflammation.

Materials and methods

The 2nd, 3rd and 4th mandibular premolars and the 2nd and 3rd maxillary premolars of seven mongrel dogs (age: 12–18 months) of both sexes were selected for study, giving a total of 140 roots that were divided into seven groups of 20 roots each.

All animals were housed in facilities approved by UNESP, Brazil. The use of animals for this research was approved by the local Ethical Committee for Animal Research and NIH Guidelines stated in 'Principles of Laboratory Animal Care' (NIH Guidelines, 1985) were followed.

The animals were anaesthetized intravenously with 3% sodium thiopental (30 mg kg⁻¹ body weight; Abbott do Brazil Ltda., São Paulo, SP, Brazil). After isolation of the dental area with a rubber dam and disinfection with 0.12% chlorhexidine digluconate, crown access was made with spherical diamond burs (Dentsply Maillefer, Ballaigues, Switzerland). The working length was determined as 2 mm short of the radiographic apex using K-files. The pulp was removed and the root canal was irrigated with 3.6 mL saline at each instrument change. The apical foramen (2 mm past the working length) was enlarged with K-files (sizes 20, 25 and 30, Maillefer) in order to standardize its diameter to enable comparative analysis and to aid the contact of LPS with the apical and periapical tissues.

Mechanical preparation was performed with K-files to the working length up to file size 40. Irrigation with 3.6 mL saline and aspiration were completed after each instrument change. A size 30 K-file was used to remove

dentine chips from the apical foramen to ensure a patent apex. The root canals were then aspirated and dried with sterile paper points and subsequently filled with EDTA for 3 min with agitation. After washing with 3.6 mL saline, the root canals were again dried and subsequently filled with a solution of LPS and saline, according to the method used previously by Silva *et al.* (2002). Briefly, under laminar airflow, 100 mg *Escherichia coli* endotoxin (lipopolysaccharide B, *E. coli* 055:B5; 9.20% lipid A; Difco–Bacto, Detroit, MI, USA) was suspended in 10 mL saline and 5 mL aliquots were kept at –70 °C until use. Coronal access cavities were then sealed with zinc oxide/eugenol cement (Dentsply Ind. Com. Ltda., Petrópolis, RJ, Brazil) over a sterile cotton pellet placed in the pulp chamber.

No control group without LPS was used in the present study because Silva *et al.* (2002) have already reported that surgical trauma causes only mild to no inflammatory reaction after 30 days.

After 10 days, the animals were again anaesthetized, the teeth were isolated as described previously and the temporary seal was removed. The 140 root canals were divided into seven groups of 20 canals each according to the irrigating solution used: group I = 1% sodium hypochlorite; group II = 2.5% sodium hypochlorite; group III = 5% sodium hypochlorite; group IV = 2% chlorhexidine digluconate; group V = saline; group VI = no irrigation, LPS remained in canal; group VII = saline during biomechanical preparation and calcium hydroxide intracanal dressing during the experimental period (Calen: 2.5 g calcium hydroxide, 0.5 g zinc oxide, 0.05 g hydrogenized colophony, 12.75 mL polyethyleneglycol 400; S.S. White Art. Dent. Ltda., Rio de Janeiro, RJ, Brazil). All irrigating solutions were obtained from the Faculty of Chemistry, UNESP, Araraquara, SP, Brazil.

Biomechanical preparation of the root canals was completed with sizes 45–70 K-files to the working length using 3.6 mL of the respective solution of each group for irrigation and then the access cavities were filled with amalgam over a glass-ionomer cement base. All variables were tested in the same animal and in the different quadrants.

After 60 days, the animals were sacrificed with an anaesthetic overdose, and the mandible and maxilla were removed. The maxilla and mandible were sectioned to obtain individual roots. These specimens were washed and demineralized with buffered EDTA in a microwave oven (Sharp Carousel, São Paulo, SP, Brazil) at 30 °C at medium/maximum power. The glass recipient with the specimens was placed in the microwave oven in another glass recipient filled with water that was surrounded by ice. The ice was changed every 10 min for 4 h day⁻¹. Complete demineralization was obtained in approxi-

mately 20 days. The specimens were then washed in running water for 24 h, dehydrated in increasing concentrations of ethyl alcohol, cleared in xylol and embedded in paraffin blocks. Serial 6- μ m sections were stained with haematoxylin and eosin and with Mallory's trichrome for histopathological analysis. Brown-Brenn stain, used for differential staining of Gram-positive and -negative bacteria, showed that there was no contamination in any experimental group.

The following parameters were evaluated and the subjective quali-quantitative results were graded according to a numerical scale: (a) intensity of inflammatory infiltrate: grade 1, absent/mild; grade 2, moderate; grade 3, severe; (b) thickness of the periodontal ligament: grade 1, normal/mildly increased; grade 2, moderately increased; grade 3, severely increased; (c) cementum resorption: grade 1, absent; grade 2, present; (d) bone resorption: grade 1, absent; grade 2, present. The analysis was performed by one examiner, who was blinded as to the group being evaluated. The parameters were evaluated in all sections and the score of each root was that that occurred with the greatest frequency in the greatest number of sections. Data were analysed with the Kruskal-Wallis statistical test and then the Dunn test for group comparison ($P < 0.05$).

Results

The frequency of histopathological parameters in the experimental groups is reported in Table 1.

Group I: 1% sodium hypochlorite

Periapical histopathological analysis of the 20 roots showed severe tissue changes (Fig. 1). On the cementum surface, there were areas of unhealed resorption and cementoblasts were absent. Extensive areas of necrotic tissue in the periapical region were observed with the

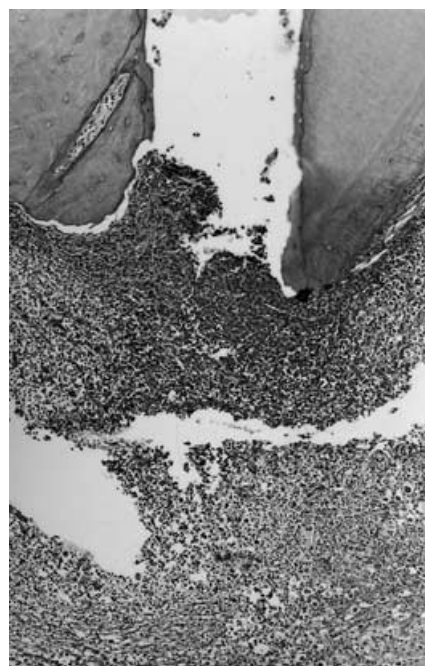


Figure 1 Group I: cementum resorption and a periodontal ligament containing mixed diffuse inflammatory infiltrate (haematoxylin and eosin, original magnification $\times 100$).

periodontal ligament severely increased in 11 specimens. The inflammatory infiltrate was mixed and diffused throughout the periapical region with generalized oedema and fibrillar dissociation classified as severe in 12 cases, moderate in six and mild in two. A large number of osteoclasts were found in the alveolar bone indicating increased bone resorption.

Group II: 2.5% sodium hypochlorite

The histological periapical alterations in the 19 roots of this group were similar to group I, with moderate/severe

Table 1 Frequency of histopathological parameters in the experimental groups

Histopathological parameters	NaOCl (1%)	NaOCl (2.5%)	NaOCl (5%)	Chlorhex (2%)	Saline	LPS	Ca(OH) ₂
Inflammatory infiltrate							
Absent/mild	2	3	7	5	0	2	16
Moderate	6	5	6	10	4	5	2
Severe	12	11	6	5	15	13	2
Periodontal ligament thickness							
Normal/mildly increased	2	3	0	3	0	2	16
Moderately increased	7	7	13	10	5	6	2
Severely increased	11	9	6	7	14	12	2
Resorption of mineralized tissues							
Cementum	18	15	14	13	15	14	3
Alveolar bone	19	19	17	18	16	17	3

inflammation, active cementum and bone resorption with necrosis and intensive vascular congestion/proliferation. The mixed periapical inflammatory infiltrate was severe in 11 cases, moderate in five and mild in three. Fifteen specimens had cementum resorption and 19 had bone resorption with a considerable quantity of osteoclasts. One root was lost during the histological processing.

Group III: 5% sodium hypochlorite

In this group, there were areas of necrotic connective tissue, oedema, haemorrhage and predominantly mononuclear inflammatory cells in the interstitial tissue of the periapical region. The inflammatory reaction was considered mild in seven cases, moderate in six and intense in six. There was intensive vascular proliferation and congestion and haemorrhagic areas. The apical cementum surface presented areas of resorption in 14 of the 19 evaluated roots and an absence of cementoblasts. There was active alveolar bone resorption, an absence of osteoblasts and a profusion of osteoclasts in 17 of the 19 evaluated roots. One root was lost during histological processing.

Group IV: 2% chlorhexidine digluconate

In 13 specimens, the cementum surface was irregular because of resorption. The interstitial tissue showed areas of necrosis and an intense concentration of mononuclear inflammatory cells within the necrotic tissue and fibrillar dissociation (Fig. 2). Intense proliferation, vascular congestion and generalized oedema were present. The inflammatory infiltrate was also mixed at a distance from the apical foramen with a predominance of macrophages and few collagen fibres in an attempt to circumscribe the inflammatory process. Osteoclasts were found in the alveolar bone with active resorption in 18 of 20 specimens.

Group V: saline solution

The 19 roots evaluated in this group presented severe periapical changes in all parameters analysed. This infiltrate was more concentrated with a considerable presence of neutrophils near the apical foramen. At a distance from the apical foramen, macrophages, intense vascular proliferation and congestion were present in the inflammatory infiltrate. In 16 specimens, the alveolar bone had a large number of osteoclasts indicating resorption.



Figure 2 Group IV: periodontal ligament containing mixed diffuse inflammatory infiltrate. Irregular collagen fibres surround the inflammatory process (haematoxylin and eosin, original magnification $\times 100$).

Group VI: LPS solution maintained in the root canal

Severe periapical changes were seen in the group in which the LPS solution was maintained in the root canal and no biomechanical preparation was performed (Fig. 3). In the interior and near the apical foramen, the invaginated interstitial periodontal tissue showed fibrillar dissociation, accentuated haemorrhage and severe and mixed inflammatory infiltrate. The inflammatory infiltrate was mild in two specimens, moderate in five and severe in 13.

Group VII: calcium hydroxide dressing (Calen)

In this group, the apical and periapical changes were considered mild (Figs 4 and 5). The inflammatory infiltrate was considered mild in 16 cases, moderate in two and severe in two roots. Near the apical foramen, the invaginated periodontal interstitial tissue showed fibrogenesis and mild inflammatory infiltrate. The periodontal ligament was of normal thickness in 16 roots, moderate in two and severe in two. The cementum presented resorption near the apical foramen in three specimens. No active alveolar bone resorption was seen and

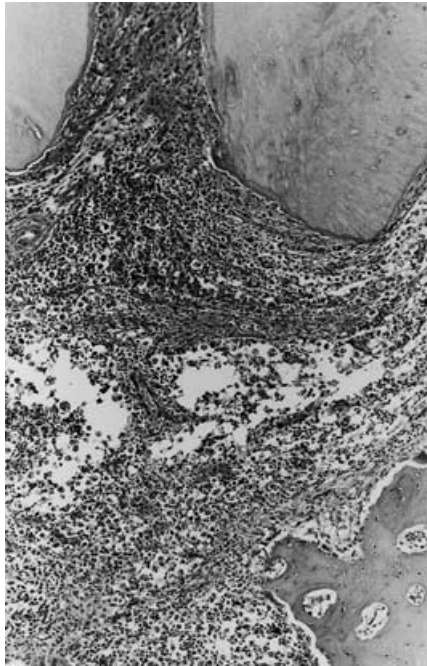


Figure 3 Group VI: apical foramen with severe inflammatory infiltrate and oedema (haematoxylin and eosin, original magnification $\times 100$).

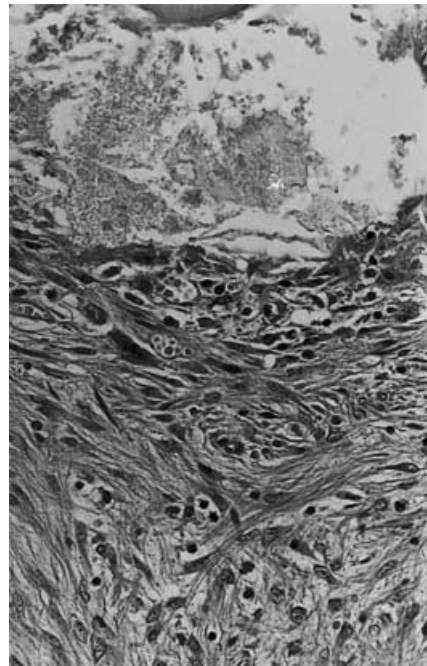


Figure 5 Group VII: an intense formation of collagen fibres and an absence of inflammatory cells around the calcium hydroxide paste (haematoxylin and eosin, original magnification $\times 400$).

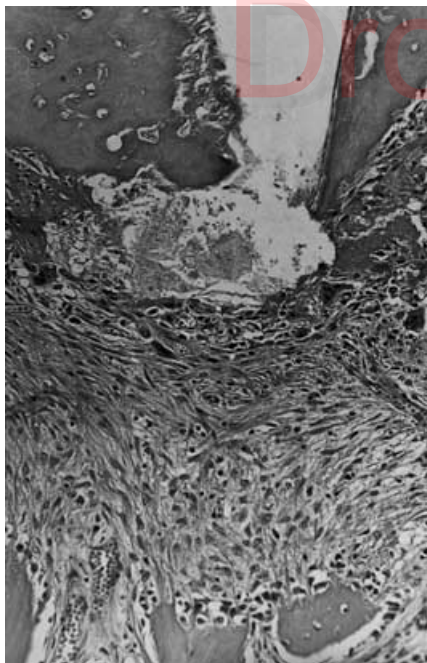


Figure 4 Group VII: calcium hydroxide paste extruded into the periapical region with mild inflammatory cell infiltrate (haematoxylin and eosin, original magnification $\times 100$).

there were osteoblasts on the surface. Only three cases presented alveolar bone resorption.

Statistical analysis

Statistical analysis of the scores given to the parameters showed that group VII was significantly different from groups I–VI, which were not significantly different.

Discussion

For histopathological evaluation, serial longitudinal sections containing all of the root apex were made, so that representative scores for each parameter could be given. Histopathological examination showed that biomechanical preparation associated with different endodontic irrigating solutions was not efficient for the inactivation of LPS-induced inflammation. Periapical inflammatory changes, and cementum and alveolar bone resorption were intense in all groups in which only biomechanical preparation was performed with results similar to group VI ($P < 0.05$), in which LPS solution was maintained in the root canal with no further biomechanical preparation. The LPS solution maintained in the root canal

during the experimental period caused an intense inflammatory reaction in the apical and periapical tissues in agreement with Mattison *et al.* (1987) after filling dog root canals with endotoxin for 4 weeks and Silva *et al.* (2002) after filling for 30 days.

Although other studies have shown that concentrated sodium hypochlorite had greater antibacterial action on Gram-negative anaerobic bacteria (Nikolaus *et al.* 1988, Georgopoulou *et al.* 1994, Yelsilsoy *et al.* 1995), in our study none of the concentrations used (1, 2.5 and 5%) reduced inflammation caused by the endotoxin, in agreement with the *in vitro* results of Buttler & Crawford (1982).

The chlorhexidine digluconate solution (2%) used during biomechanical preparation was also ineffective in eliminating the effects of the endotoxin, confirming results of Aibel & Stevens (1999) in an *in vitro* study with a 1.2% chlorhexidine concentration.

Quantifying the effect of irrigating solutions and calcium hydroxide on endotoxin by means of free fatty acid release, Buck *et al.* (2001) reported that 0.12% chlorhexidine solution, 2.6% sodium hypochlorite or the association of both were not efficient. However, the use of calcium hydroxide dressings for 1-, 2- or 5-day hydrolyzed bacterial LPS.

Silva *et al.* (2002) reported no periapical inflammation after maintaining dog root canals filled with saline for 30 days. Dahlén *et al.* (1981) maintained monkey root canals empty or filled with saline for 3–7 months and reported an absence or only a mild periapical inflammatory reaction. These two studies did not involve filling the root canal, demonstrating that maintaining the empty root canal or the root canal filled only with saline did not cause severe periapical reactions.

In this study, after filling the root canals with endotoxin and biomechanical preparation with different irrigating solutions, these solutions were maintained in the root canals during the experimental period. Tanomaru Filho *et al.* (2002) reported unsatisfactory periapical repair after a one-session endodontic treatment using 5.25% sodium hypochlorite or 2% chlorhexidine digluconate during biomechanical preparation in dog teeth with radiographically visible periapical lesions. When a calcium hydroxide dressing was used, apical and periapical repair were satisfactory, similar to the results of this study.

Lipid A molecule hydrolysis was observed *in vitro* by Safavi & Nichols (1993) and they concluded (Safavi & Nichols 1994) that this results in the formation of fatty acids and amino-sugars that are atoxic components.

Barthel *et al.* (1997) evaluated the neutralization capacity of calcium hydroxide on the biological activity of *Escherichia coli* endotoxins quantifying the TNF- α liberation in human monocytes, concluding that calcium hydroxide neutralized bacterial LPS *in vitro*. Similar results were obtained by Olsen *et al.* (1999) using monocyte release of IL-1 β .

Silva *et al.* (2002) verified *in vitro* the inactivation of lipid A after root-canal filling of dog teeth with an LPS solution and calcium hydroxide and observed satisfactory histopathological results in apical and periapical tissues.

Bacterial LPS is important in teeth with periapical lesions. Its effects on periapical tissues and the limited number of agents to effectively inactivate it during endodontic treatment support the routine use of calcium hydroxide during endodontic treatment of teeth with apical periodontitis.

Conclusion

This study showed that biomechanical preparation with the different irrigating solutions did not inactivate the effects of the endotoxin but the calcium hydroxide intracanal dressing did appear to inactivate the effects induced by the endotoxin *in vivo*.

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